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ESX encodes an Ets family transcription factor gene that is potentially important in breast cancer because the ESX genomic region (chromosome 1q32.1) is amplified in 50% of early breast cancers and ESX mRNA is over-expressed in human breast ductal carcinoma in situ (DCIS). However, the precise molecular mechanism by which ESX mediates breast cell transformation remains unknown. We have now completed the key milestones originally proposed. Specifically, we have demonstrated that stable expression of HA-tagged or GFP-tagged ESX transforms the human, non-transformed MCF-12A cell line (which fails to express endogenous ESX). Moreover, we have documented that the subcellular localization of Esx is cytoplasmic, and that it is this subcellular localization that is required for transformation. Indeed, nuclear localization appears to mediate apoptosis. We have excluded the key transcriptional motifs as being required for transformation, and have mapped the transforming Esx subdomain to a small, acidic region. Taken together, these data reveal that Esx is not functioning as a transcription factor to transform MCF-12A mammary cells, but rather that ESX functions primarily via cytoplasmic mechanisms. Finally, we demonstrate that endogenous Esx protein is localized in the cytoplasm in human breast cancer specimens. In summary, these are extremely novel results and challenge dogma that Ets factors must always function in the nucleus as transcription regulators.

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INTRODUCTION

The Ets family of transcription factors contains several members that are important components of the cellular pathways leading to tumorigenesis (1). For example, several Ets members are downstream targets of oncogenic Ras (2); dominant-negative Ets reverses the transformed phenotype (3,4); and, Ets proteins have been shown to regulate a repertoire of genes that govern cellular survival, proliferation and migration (1,6). Moreover, several Ets factors have been implicated in breast cancer (1,6). However, the ability of Ets factors to transform human breast cells, the identity of the precise Ets factor required for breast cell transformation, and the molecular mechanism by which such an Ets factor mediates breast cell transformation, all remain unknown. The ESX gene is an Ets member that is particularly relevant to breast cancer. ESX is located on chromosome 1q32.1, in a region that is amplified in 50% of early breast cancers. ESX mRNA is over-expressed in human breast ductal carcinoma in situ (DCIS) (7-9). Also, there is a positive feedback loop between the HER2/neu proto-oncogene and ESX, in that HER2/neu activation induces ESX expression, while ESX activates the HER2/neu promoter via a putative ESX DNA binding site (7-9). Finally, HER2/neu and ESX expression levels are positively correlated in human breast cancer cell lines (7-9). Based on these observations, we have chosen to determine whether ESX is capable of transforming immortalized, but non-transformed MCF-12A human breast cells, and to determine the precise mechanism(s) by which ESX transforms these human breast epithelial cells.

BODY

Task 1: To define the modular structural domains of ESX (\pm DNA) using Cleveland and peptide sequencing analysis.

As noted in our previous Progress Reports, and now presented in a publication (10), we have fully characterized an HA-tagged ESX mammalian expression construct, showing that HA-ESX is expressed as a protein of about ~47 kDa in transiently transfected HeLa and MCF-12A cells. We also characterized the transcription potency and promoter specificity of HA-ESX (10). Taken together, these data revealed that HA-ESX differentially regulates several breast-cancer relevant gene promoters, in particular collagenase and HER2/neu.

We also generated ESX as a fusion protein with GST, and we have optimized conditions to express recombinant GST-ESX in bacteria and to purify it to homogeneity. Having made GST-ESX containing a thrombin cleavage site between GST and ESX, we also optimized conditions for cleavage of ESX from GST-ESX immobilized on glutathione beads. While this approach has been releasing intact ESX, the amounts released were initially very low (~2-5% of total). We have now optimized the expression and purification of ESX devoid of the GST leader in an efficient manner. Finally, we have also subcloned defined, but overlapping, regions of ESX into a bacterial expression vector with the long term goals of producing recombinant ESX fragments as affinity matrices for protein purification (see Task 3) and for region-specific antibody development.

Using the highly purified, full-length recombinant GST-ESX, we generated rabbit polyclonal anti-ESX antibodies, in collaboration with ABR, Inc., in Golden, CO, as originally proposed.

These antibodies, which are very important reagents, are now commercially sold by ABR to the entire scientific community, thus providing an invaluable research tool to all breast cancer investigators. We have characterized these anti-ESX antibodies and shown that they recognize a ~47 kDa nuclear protein that is expressed in T47D breast cancer cells. Importantly, we also documented that ESX is not expressed in the non-transformed human MCF-12A mammary cell line (11). Additionally, these anti-ESX antibodies have already been very useful in measuring ESX protein expression levels in the transcription and transformation assays, as described above. We also initiated a new project with these anti-ESX antibodies and we have optimized the chromatin immunoprecipitation (ChIP) assay to show that ESX actually binds to certain cellular target genes *in vivo*, during the initial stages of ESX expression (12).

We have used these anti-ESX antibodies in immuno-histochemical (IHC) analysis of ESX expression in primary human breast cancer specimens and immuno-cytochemical (ICC) analysis of T47D (ESX⁺), MCF-12A (ESX⁻) and MCF-12A (ESX⁺) human mammary cell lines (12). In collaboration with Dr. Menakshi Singh, in our Pathology Dept., we show that endogenous ESX is primarily located in the cytoplasm in primary human breast cancer specimens and T47D breast cancer cell line (12). By contrast, no ESX protein was detected in the control MCF-12A cells, whereas ESX was detected primarily localized to the nucleus in MCF-12A cells transiently transfected with an HA-ESX expression vector (12). These data suggest differential localization of ESX in malignant (cytoplasmic) cell lines and tissues vs nonmalignant (nuclear) cell lines, and our most recent report verifies this point as a novel mechanism of mammary cell transformation mediated by a putative transcription factor.

Finally, while we initially pursued the Cleveland digestion protocol \pm DNA, as originally described in our proposal, as our studies progressed we realized that ESX transforms cells via a cytoplasmic mechanism and not via a nuclear, transcription-mediated mechanism. For this reason, we halted these studies for now, and decided to pursue the more physiologically-relevant studies in Tasks 2 and 3.

Task 2: To define the modular domain of ESX required for breast cell transformation.

These studies turned out to be the most informative of all, and the results are strikingly novel! We have made several important discoveries about the role of ESX in breast epithelial cell proliferation and transformation, and the data are detailed in three manuscripts (10-12). First of all, we have completed the preliminary studies showing that transient expression of HA-ESX and HA-VP16-ESX in MCF-12A cells results in increased colony formation (10). Using a colony formation assay, we found that HA-ESX and HA-Ets-2 mediated MCF-12A cell colony formation rates that approached those generated by oncogenic V12 Ras, whereas empty vector had a negligible effect. By contrast, in immortalized and transformed T47D breast cancer cells, which express abundant amounts of HER2/neu and ESX, we found that anti-sense and dominant-negative HA-ESX inhibited T47D colony formation, whereas control vector allowed formation of many colonies.

In the second report (11), we show that we have established a number of MCF-12A cell lines stably expressing either vector control HA-ESX, HA-VP16-ESX, HA-ETS-2, or V12-Ras. We chose the MCF-12A cell line because it is immortalized, but nontransformed, and importantly these cells fail to express endogenous ESX protein. We used pCGN2-HA-Ets-2 and pSVRas expression vectors as positive controls for transformation. Stable expression of ESX induced EGF-independent proliferation, serum-independent MAPK phosphorylation and growth in soft agar. Additionally, stable ESX expression conferred increased cell adhesion, motility and invasion in two-dimensional and trans-well filter assays. An epithelial to mesenchymal morphological transition was noted in stable ESX cells. In three-dimensional cultures, parental and control (pCGN2) cells formed highly organized duct-like structures with evidence of cell polarity, ECM adhesion-dependent proliferation and cell survival, and lack of cellular invasion into surrounding matrix. Remarkably, the ESX stable cells formed solid, disorganized structures, with lack of cell polarity and loss of dependence on ECM adhesion for cell proliferation and survival. In addition, ESX cells invaded the surrounding matrix, indicative of a transformed and metastatic phenotype. The positive control cell lines, HA-Ets-2 and V12Ras, also increased adhesion, motility and invasion, while displaying differences in cellular morphology. Finally, the negative control (pCGN2) cells lacked any evidence of the transformed and EMT phenotypes.

These studies have been completed in collaboration with Dr. Pepper Schedin of the AMC Cancer Center here in Denver, and this Ms. detailing these results has been accepted by *Oncogene* (see Appendix). This study establishes ESX and Ets-2 as putative oncogenes capable of conferring the transformed phenotype to otherwise normal MCF-12A human mammary epithelial cells.

In the third paper, we have focused on mapping the subdomain of ESX that is necessary and sufficient for transforming MSF-12A cells, using GFP-fusions of ESX sub-regions and colony formation in soft agar as the key assays. Specifically, since we had not been able to detect HA-ESX protein in the MCF-12A cells stably transfected with the HA-ESX expression vector, we were unable to determine its subcellular localization, despite the fact that HA-ESX actually transformed these human mammary cells. However, the cytoplasmic localization of ESX in the T47D breast cancer cell lines and primary tissues forced us to reconsider our original hypothesis and also forced us to more carefully analyze the subcellular localization of ESX. To this end, we constructed a GFP-ESX fusion and generated multiple distinct pools of MCF-12A cells stably expressing this construct. Real-time GFP-ESX expression in living cells was followed on a daily basis using fluorescence microscopy during the G418 selection process. These studies revealed that GFP-ESX is initially robustly expressed in the nucleus in most cells, and in the cytoplasm in fewer cells. After about 28-30 hours, the cells expressing GFP-ESX in the nucleus die by apoptosis, and the population expressing GFP-ESX in the cytoplasm persist and become the dominant and ultimately transformed cell population.

To map the region of ESX required for MCF-12A cell transformation, we fused various ESX constructs that had been internally deleted of key functional domains (eg, Pointed, TAD, acidic/Sox and Ex7/NLS domains) to GFP. The data reveal that deletion of any domain involved with either nuclear localization (Ex7/NLS) or transcription (Pointed or TAD), did not

interfere with the transforming potential of ESX in the soft agar colony formation assay. By contrast, internal deletion of the 40-AA acidic/Sox domain resulted in complete loss of ESX's transforming ability. Furthermore, adding a strong NLS to either the WT or the Ex7/NLS-deleted ESX constructs resulted in loss of transformation ability. Finally, to determine whether the 40-AA acidic/Sox domain is necessary and sufficient for transformation, we fused this short region alone to GFP and documented that it resulted in robust colony formation in soft agar in a manner equivalent, if not better, than the full-length ESX version. Furthermore, appending an NLS signal to the 40-AA acidic/Sox domain reversed its transforming capability, again proving that nuclear localization is incompatible with its ability to transform MCF-12A cells (12).

Task 3: To identify the proteins that associate with the ESX transforming domain using MALDI-TOF.

As noted above, our more recent discovery that endogenous ESX is localized primarily to the cytoplasm in human breast cancer specimens and in the T47D breast cancer cell line, and that cytoplasmic expression of the 40-AA transforming ESX motif is sufficient for transformation, we have had to reassess our original hypothesis. We originally hypothesized that ESX main function was as a transcription factor, however, it has now become clear that it functions primarily in the cytoplasm. Thus, the overall approach to this Aim remains essentially unchanged, except that we will focus on purifying cytoplasmic proteins that bind to the ESX transforming 40-AA domain. In this regard, we have already generated multiple GST-ESX fusion constructs, including one that contains the 40-AA acidic Sox domain. Moreover, we have initiated affinity chromatography purification steps using this GST-40AA(Acidic/Sox domain), in order to purify cytoplasmic proteins that bind to this transforming motif. Preliminary purification studies completed during the "no cost extension" period revealed three bands, elongation factor-1 alpha (eEF-1 α) and gamma-actin (γ -actin). The biological relevance of these two proteins in the SOX-mediated transformation process is currently being evaluated.

KEY RESEARCH ACCOMPLISHMENTS

- ◆ Establishment of multiple pools of MCF-12A cells stably expressing HA-ESX, HA-VP16-ESX, HA-Ets2, V12-Ras or vector control.
- ◆ Documenting that ESX and Ets-2 function as oncogenes and transform MCF-12A human mammary cells, resulting in a functional epithelial-to-mesenchymal transformation (EMT) phenotype.
- ◆ Showing that endogenous ESX is localized to the cytoplasm in human breast cancer specimens and T47D breast cancer cell lines, using IHC and ICC, respectively.
- ◆ Establishment of GFP-ESX stable pools in MCF-12A cells and discovering that GFP-ESX is primarily localized in the cytoplasmic subcellular region.
- ◆ Generating GFP-ESX internal deletions and defined domain fusion constructs, and establishing pools of MCF-12A cells stably expressing each of these constructs.
- ◆ Using these constructs to show that cytoplasmic expression of a 40-AA acidic/Sox domain is necessary and sufficient for MSF-12A cellular transformation.

REPORTABLE OUTCOMES

Abstracts:

- (1) Identification of ESX-Regulated Genes that Promote Breast Cell Transformation by cDNA Microarray Analysis. G.J. Cappetta, Kristin Eckel, Arthur Gutierrez-Hartmann, University of Colorado Health Sciences Center, Denver, CO. National Endocrine Society Meeting, Denver, CO, June, 2001.
- (2) Analysis of ESX Function in the Development of Human Breast Cancer. J.D. Prescott, A. Gutierrez-Hartmann, University of Colorado Health Sciences, Denver, CO. National Endocrine Society Meeting, Denver, CO, June, 2001.
- (3) The ESX Transcription Factor in Human Breast Cancer: Localization of the ESX Protein in Human Breast Epithelial Cells and Identification of Her-2 as an ESX Target Gene. J.D. Prescott, M. Singh, A. Gutierrez-Hartmann, University of Colorado Health Sciences, Denver, CO. National Endocrine Society Meeting, San Francisco, CA, June, 2002.
- (4) ESX induces transformation and functional epithelial to mesenchymal transition in MCF-12A mammary epithelial cells. P.J. Schedin, K.L. Eckel, S.M. McDaniel, K.S. Brodsky, J.J. Tentler, A. Gutierrez-Hartmann, AMC Cancer Research Center, Denver, CO; University of Colorado Health Sciences Center, Denver, CO. American Association for Cancer Research Meeting, San Francisco, CA, 2002.
- (5) The Ets Transcription Factor ESX Directly Activates Expression of the HER-2 Proto-oncogene. J.D. Prescott & A. Gutierrez-Hartmann, University of Colorado Health Sciences, Denver, CO. San Antonio Breast Cancer Symposium Abstract, 2002.
- (6) Cytoplasmic expression of ESX is required for MSF-12A transformation. J.D. Prescott, K. Koto, M. Singh, A. Gutierrez-Hartmann, University of Colorado Health Sciences Center, Denver, CO. American Association for Cancer Research Meeting, Washington, DC, 2003.

Manuscripts

- (1) Eckel KL, Tentler JT, Cappetta GJ, Diamond SE and Gutierrez-Hartmann A. (2003) The epithelial-specific ETS transcription factor ESX/ESE-1/Elf-3 modulates malignancy-associated gene expression and breast cell growth. *DNA & Cell Biol.* 22:79-94..
- (2) Schedin PJ, Eckel KL, McDaniel SM, Brodsky KS, Tentler JT, Prescott JD and Gutierrez-Hartmann A. ESX Induces transformation and functional epithelial to mesenchymal transition in MCF-12A mammary epithelial cells. *Oncogene* 23:1766-1779, 2004.
- (3) Prescott JD, Koto K, Singh M, Gutierrez-Hartmann A. Cytoplasmic expression of an acidic 50-amino acid region of the ETS transcription factor ESE-1 defines a novel mammary transformation mechanism. *Mol Cell Biol* 24:5548-5564, 2004.
- (4) Coletta RD, Jedlicka J, Gutierrez-Hartmann A, Ford HL. Transcriptional control of the cell cycle in mammary gland development and tumorigenesis. (Invited review). *J Mamm Gland Biol* 9:39-53, 2004.

Reagents Developed

- (1) Rabbit polyclonal anti-ESX antibodies now commercialized by ABR, Inc., Golden, CO.

Funding applied for and received based on this work

(1) J.D. Prescott, National DOD Pre-doctoral Award, 6/2001: Awarded, 6/2002.

CONCLUSIONS

The 3-year support provided by the DOD/DAMD Idea Award has been critical in allowing us to pursue ESX as a putative mediator mammary cell transformation and of ESX as clinical target in breast cancer. Using this support, we have made several critical and very innovative discoveries regarding ESX and mammary tumorigenesis that is very likely to impact the clinic. Specifically, we have shown that while ESX is an Ets-family transcription factor that is capable of acting as a nuclear regulator of gene expression in transient transfection reporter assays, that this is not the physiologically-relevant mode of ESX action. We used a variety of techniques to show that stable expression of ESX in MCF-12A cells results in all the hallmarks of cellular transformation and an epithelial-to-mesenchymal transition. Furthermore, we carefully showed that stable expression occurs in the cytoplasm and that nuclear targeting of ESX (or its subdomains) reverses the transformed phenotype. Finally, we mapped the transforming ESX domain to a 40-AA highly acidic motif that has been reported to be similar to the Sox domain, and we showed that the cytoplasmic expression of this motif is necessary and sufficient for MSF-12A cellular transformation. While we do not yet understand the mechanism by which this 40-AA domain mediates cellular transformation, we do know that it is initially via cytoplasmic effectors. This is in striking contrast with its known structure as a transcription factor of the Ets-family, wherein it would be expected to function via regulation of promoter activity. Finally, given that endogenous ESX is expressed cytoplasmically in human breast cancer, these issue become very clinically relevant. Ultimately, we anticipate that these studies will provide a novel marker and several new drug targets to use in our battle against breast cancer.

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Appendices: None